Nomination to ICCVAM: BoCell™ A Cell-based Assay for Botulinum Neurotoxin A Detection.

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Preamble: BioSentinel is submitting nominations for three different product types- the BoTest™ BoNT Detection Kits, the BoTest™ Matrix BoNT Detection Kits, and the BoCell™ A Cell-based assay. These products differ in their BoNT sensitivity and tolerance to complex matrices. While an independent evaluation of each assay is required, these products should be considered a suite of solutions for detecting and quantifying BoNT. Thus, the products can be used in combination to meet criteria necessary for improved disease diagnosis, improved drug product production and development, and reduced animal testing.

1.0 Introduction and Rationale for the Proposed Test Method

1.1 Introduction

1.1.1 **Background.** The BoCell™ A assay is a cell-based assay for the detection of botulinum neurotoxin serotype A (BoNT/A). The assay relies on an engineered cell line that expresses a reporter that responds to intracellular BoNT proteolytic activity. BoNT is only detected if the toxin enters the cells through the toxin's cell receptor binding, uptake, and translocation activity. The BoCell A assay mimics the natural cell biology of BoNT/A taking into account all of BoNT/A's native cellular functions. Thus, the BoCell™ is intended to be used as a direct replacement for the mouse bioassay.

The fluorescent reporter used for the BoCell™ assay was invented by Dr. Edwin Chapman's group at the University of Wisconsin and reported on 2004[1]. Dr. Chapman described a reporter that utilizes full-length SNAP-25 fused to two fluorescent proteins (**Fig. 1**). The reporter could be transiently expressed in living cells and BoNT/A activity detected by either a loss if Förster Resonance Energy Transfer (FRET) or by destruction of the C-terminal fluorophore.

The BoCell™ technology was subsequently licensed by BioSentinel who began operations in June 2007 and initiated a R&D program to fully develop the BoCell™ assay. Specific goals included improving the cellular reporters' performance, increasing assay sensitivity, and developing engineered cell lines that stably express the reporter. BioSentinel has since developed a cell line that stably produces the reporter over many passages and demonstrated that the BoCell cell-based assay can be used with a fluorescent microplate reader. BioSentinel intends to launch the BoCell™ A Cell-based Assay for BoNT/A Detection in Q3 2011 with the assay sold by licensing agreement. BioSentinel is currently developing assays for serotypes other than BoNT/A.

The BoCell assays were developed because there is currently no commercially available cell-based assay for the detection and quantification of BoNT. A cell-based assay, used in conjunction with other analytical methods, is the only type of assay that is likely to provide complete replacement of the current test method,

the mouse bioassay, for drug product manufacturing, BoNT diagnostics, and biodefense testing. The BoCell™ assay, like the mouse bioassay, takes into account all of the toxin's cellular activities (**Fig. 2**). Unlike the mouse bioassay, the BoCell™ offers high throughput assays and minimal equipment investments. The BoCell, however, is not as sensitive as the mouse bioassay.

1.1.2 **Peer review to date**. BoCell™ is currently under review at the FBI CBRN Sciences Unit.

The BoCell™ assay has not undergone any formal government panel peer review.

1.1.3 **Confidential information.** No Information contained in these documents is considered confidential.

1.2 Scientific basis for the proposed test method

1.2.1 **Purpose and mechanistic basis.** The BoCell™ A assay detects BoNT/A protease activity but only after BoNT/A has been internalized by cells via BoNT/A's receptor bind, uptake, and translocation activities (**Fig. 2**). The assay was designed to provide an easy, medium to high throughput means to quantify BoNT preparations and to discover new BoNT therapeutics. Currently, BioSentinel offers one cell-based assay specific for BoNT/A. Assays against the other five serotypes are in development.

The BoCell™ assay relies upon an engineered cell line that expresses a reporter that contains full-length SNAP-25 flanked by cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP; **Fig. 1**). When the engineered cells are incubated with BoNT/A, BoNT/A is internalized resulting in the release of the BoNT/A light chain into the cytosol. The BoNT/A light chain then cleaves the reporter resulting in the release of a C-terminal reporter fragment into the cytosol that contained resides 198 – 206 of SNAP-25 and YFP. That fragment is rapidly destroyed by the cell resulting in a loss of YFP fluorescence. Data is captured by measuring the total YFP emission of the cells using directly excited YFP; **no FRET fluorescence is used in the assay.** Directly CFP fluorescence is also collected to normalize for cell density and fluorescence expression. Thus, YFP emissions are divided by CFP emissions giving a ratiometric assay read-out.

The BoCell™ assay is completed in a 96-well plate using a fluorescence microplate reader; no specialized imaging equipment is required. The assay does require incubating the cells with BoNT/A for 24 – 72 h to reach the desired sensitivity. BoNT/A sensitivity in the picomolar range requires a 72 h incubation period. The BoCell assay is ~2 – 3 orders of magnitude less sensitive that the mouse bioassay.

1.2.2 **Similarities & differences between test method and mode of action.** BoNTs paralyze neurons and cause botulism by cleaving proteins that are essential for regulated neurotransmitter release. The BoCell™ assay detects and quantifies this endopeptidase activity using reporters that are modeled after the native targets of BoNT. Thus, the BoCell™ Matrix assays account for BoNT biological activity rather than simply toxin mass.

The BoCell™ assay does take into account, unlike the BoTest™ and BoTest™ Matrix assays, for the ability of BoNT to bind to and enter neurons. Because the BoCell™ reporter is expressed inside cells, BoNT/A must gain access to the cell cytosol via its receptor binding, uptake, and translocation activities (**Fig. 2**). Thus, the BoCell™ assay is the only assay with commercial availability that mimics BoNT's natural cell biology.

1.2.3 **Range of testable substance.** The BoCell™ assay has been tested with purified preparations of BoNT/A in various tissue culture media.

2.0 Test Method Protocol Components.

A DETAILED PROTOCOL IS CONTAINED IN ATTACHMENT 8.1.

2.1 **Protocol overview.** The BoCell™ assay requires basic tissue culture skills to complete. Engineered cells are maintained with a 3-day expansion and passaging protocol. When an assay is required, cells are collected and plated into wells of a 96-well plate and allowed to expand for 24 h. The cells are then incubated with samples containing BoNT/A for 24 – 72 h to reach desired sensitivity. Cells are washed with PBS (to reduce background) before collecting fluorescence emissions. Data is captured using a fluorescence microplate reader capable of detecting emissions at two wavelengths. The fluorescence emissions are converted into a ratiometric value and plotted as a function of BoNT concentration or time. Unknowns can be quantified using an appropriate BoNT standard and basic curve fitting software.

2.2 Protocol details

2.2.1 **Materials, equipment, and supplies needed.**

- BoCell™ A cell line
- Commercially available tissue culture medium and antibiotics
- Fetal Bovine Serum
- PBS
- Various plastics (conical tubes, basins, microcentrifuge tubes, etc)
- Fluorescence microplate reader with 434 nm excitation, 470 nm emission, and 526 emission filters
- Black-sides, clear-bottom 96-well microtiter plates with covers
- CO_2 Incubator to 37 ^{0}C, 5% CO_2
- Microplate washer (optional)
- BoNT/A
- Biological Safety Cabinet
- Pipettes and tips for 1 μl – 200 μl dispensing

2.2.2 **Dose selection procedure.** Due to its real-time nature, assay sensitivity is time tunable with an expected range of quantifiable BoNT concentrations between 50 pM and >10 nM when including an appropriate standard curve containing 0.5 log BoNT dilutions.

2.2.3 **Endpoint measured.** For each data capture event, emissions are collected at 526 nm with 500 nm excitation and at 470 nm with 434 nm collection. The relative fluorescence unit (RFU) value at 526 nm is then divided by the RFU value of 470, yielding a ratiometric value. The ratiometric value is then compared

to the ratiometric values obtained using a standard BoNT curve.

2.2.4 **Known limits of use.** BioSentinel has only tested a limited set of matrices; some matrices may interfere with assays.

2.2.5 **Nature of response assessed**. The BoCell™ assay detects and quantifies BoNT proteolytic (direct), cell binding (indirect), uptake (indirect), and translocation (indirect) activities.

2.2.6 **Appropriate positive and negative controls**. Each assay should contain a standard curve composed of known BoNT concentrations. A negative control will contain no BoNT in a buffer ideally identical to the unknowns.

2.2.7 **Acceptable range of positive and negative controls.** The standard curve should contain concentrations of BoNT that give a dose-response curve that clearly demonstrates assay saturation (complete reporter cleavage) and no activity (reporter intact). Clear evidence of both is required for accurate curve fitting of the standard curve and solving unknowns.

2.2.8 **Methods to analyze the resulting data.** Ratiometric values obtained from the standard curve are plotted versus the known BoNT concentration contained in each standard sample. The data is then fitted with equation Y = Bottom + (Top – Bottom)/(1+$10^{[(\text{LogEC}_{50}\text{-x})*\text{Hill slope}]}$), where X is the logarithm of BoNT concentration and Y is assay response, yielding an EC_{50} value. The fit is then used to solve for unknown concentrations.

2.3 Basis for test method system. The test system was selected because it is the only commercial-ready BoNT detection method with picomolar BoNT sensitivity that takes into account all BoNT cellular activities. In addition, the method does not require the use of animals.

2.4 Proprietary components and their integrity over time and production runs. BioSentinel manufactures the BoCell™ A assay in its entirety. BioSentinel maintains manufacturing records that document all raw materials (manufacturer, part and lot numbers), each step in the manufacturing process, and quality control steps taken to ensure expected performance. In addition, BioSentinel's quality assurance program securely maintains all manufacturing records, standard operation procedures, and employee training. Finally, BioSentinel tests product integrity with accelerated and long-term stability experiments.

3.0 Product Performance and Stability.

3.1 Product performance

3.1.1 **Figure 3** shows how the BoCell™ cells' fluorescence emissions change in response to BoNT/A. Data was collected with a fluorescent microscope to illustrate how changes occur over the entire cell population.

3.1.2 **Figure 4** demonstrates that the BoCell™ assay can be completed using both high cost (microscope and high content imager) and low cost (microplate reader) instruments.

3.1.3 **Figure 5** shows the expected statistical performance of the BoCell™ A Cell-based Assay. These data were collected with a microplate reader. Limits of

detection (LOD) and quantification (LOQ) were calculated from the curve fit and assay responses that are 3 or 10, respectively, standard deviations from control responses in the absence of BoNT. Note that these data will be dependent on the activity of a particular BoNT/A preparation.

3.2 Product qualification and stability

3.2.1 **Figure 6** demonstrates the selection of the engineered cell line that became the BoCell™ cell line and its stability over passage. Note that CL1 and CL2 lose assay performance over passage, likely due to a loss of reporter expression. CL3 and 4 maintained performance over 20 passages. CL4 was eventually selected for BoCell™ A Cell-based Assay development.

3.2.2 **Figure 7** shows that only intact BoNT can elicit a response in the BoCell™ assay. BoNT/A holotoxin contains both the heavy-chain and light-chain. The heavy-chain is responsible for BoNT's cell receptor binding, uptake, and translocation activities. The light-chain is responsible for BoNT's proteolytic activity.

3.2.3 **Figure 8** demonstrates that BoNT/A uptake in the BoCell™ assay can be blocked using a competitor for cell receptor binding. HcR/A is a recombinant BoNT fragment containing the receptor binding domain of BoNT/A but no other activity domains.

4.0 Other scientific reports or reviews.

4.1 Similar methods. A sensitive primary neuron assay for BoNT detection was recently reported, but it depends on animal use for neuron extraction and uses low-throughput protein electrophoresis and blotting techniques[2;3].

5.0 Animal Welfare Considerations

5.1 Refinement, Reduction, and Replacement. The BoCell™ Cell-Based Assay does not require the use of animals and represent the only commercially available, animal-free assay that accounts for all of BoNT's proven cellular activities. Thus, the BoCell™ assay has the potential to greatly reduce animal assay use in the manufacturing of drug products, the detection of disease, and for testing suspect substances (biodefense).

The BoCell™ assay is not as sensitive as the mouse bioassay by 2-3 orders of magnitude. However, for many applications, the BoCell™ assay's sensitivity meets requirements (in-process manufacturing, biodefense). For instances where more sensitive assays are required, the BoCell™ assay can be used in conjugation with the BoTest™ or BoTest™ Matrix assays. Given an application, the combination of these three assays can be tailored to meet a desired endpoint.

6.0 Practical Considerations

6.1 Transferability. The BoCell™ assay can be carried out in any laboratory with basic tissue culture capabilities. A biological cabinet and CO_2 incubator would be required. The only other specialized equipment requirement is a fluorescence microplate reader with the ability to measure emissions at two wavelengths. A microtiter plate washer with cell washing capabilities will improve throughput but is not required. Other equipment and reagents required are typically found in most laboratories (e.g. pipettes, nanopure H_2O, etc) or can be ordered from vendors (tissue culture media, antibiotics).

The current reference test method, the mouse bioassay, requires specialized mouse handling equipment often housed in a space with purpose-designed air handling infrastructure. For this reason, the mouse bioassay is currently only carried out at a handful of state, federal, and commercial labs. The BoCell™ assay can be transferred to a much large segment of Federal and State testing laboratories.

6.2 **Training.** The BoCell™ Cell-based Assay requires basic tissue culture skills to maintain the BoCell™ cell line and run the assay. The training level is similar to what is required to maintain a typical adherent cell line, such as HEK293 or PC12 cells. Some additional instruction is required to maximize assay reproducibility and can be completed in the form of an instructional presentation or one-day on-site training session. In contrast, the mouse bioassay requires training and certification is animal handling and extensive experience with animal injections, observing animals for the clinical symptoms of botulism, and transforming data into a useful predictor of toxicity.

6.3 **Cost Considerations.** Costs cannot be estimated without a specific endpoint and application to consider. The BoCell™ Cell-based Assay will be a licensed product and licensing terms will be largely dependent on the licensing organization and the organization's intended use. Running costs will be in line with maintaining a typical adherent cell line.

Quantifying a sample using the mouse bioassay would be much more expensive. For regulatory approved, drug product-grade mouse bioassays, a single quantification assay can require as many as 200 – 300 mice at an estimated cost of $6,000 if completed at a commercial laboratory operating under good laboratory practices. Cost savings are minimal when multiple samples are quantified at the same time as each sample would require the same number of injections to maintain assay accuracy and precision measures.

6.4 **Time considerations.** Depending on the number of samples and the desired sensitivity, the BoCell assay takes 24 – 96 h to complete. A typical mouse bioassay protocol takes 48 – 96 h to complete.

7.0 References.

1. Dong, M., Tepp, W. H., Johnson, E. A. & Chapman, E. R. (2004). Using fluorescent sensors to detect botulinum neurotoxin activity in vitro and in living cells. *Proc Natl Acad Sci U S A* **101**, 14701-6.
2. Pellett, S., Tepp, W. H., Clancy, C. M., Borodic, G. E. & Johnson, E. A. (2007). A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies. *FEBS Lett* **581**, 4803-8.
3. Pellett, S., Tepp, W. H., Toth, S. I. & Johnson, E. A. (2010). Comparison of the primary rat spinal cord cell (RSC) assay and the mouse bioassay for botulinum neurotoxin type A potency determination. *J Pharmacol Toxicol Methods* **61**, 304-10.

8.0 Supporting Materials (Appendices).

8.1 BoCell™ A Cell-based Assay Protocol.

8.2 Figures, legends, and tables.

Attachment 8.1. BoCell™ A Cell-based Assay Protocol.

SOP NO.: GL1042

Title: Exposure of mammalian cells to BoNT/A in a 96 well plate format

1. PURPOSE AND DEFINITIONS:

1.1 The purpose of this SOP is to give instructions on the treatment of mammalian cells with BoNT/A in a 96 well plate.

2. SCOPE:

2.1. This SOP applies to the addition of BoNT/A to mammalian cells grown in 96 well plates.

3. RESPONSIBILITY:

3.1 Any lab member who wishes to treat mammalian cells with BoNT/A.

4. SAFETY PRECAUTIONS:

4.1 This procedure requires the use of botulinum neurotoxins (BoNT). BoNT is extremely toxic.

a. All lab personell must read Safety SOP GL1005 and be trained for the handling of BoNT.

b. All procedures requiring manipulation of BoNT should be carried out in a chemical or biological hood.

c. Lab personnel are required to wear safety goggles and nitrile gloves at all times.

d. While carrying out this procedure, the lab door must be closed and locked at all times.

e. All BoNT-containing sample must be disposed of by SOP GL1009.

5. MATERIALS:

5.1 Direct materials: None

5.2 Indirect materials:

Material	Vendor	Part Number	Lot Number
Greiner TC 96 well plates, black with clear bottoms	VWR	82050-748	
MEM medium	ATCC	30-2003	
Fetal bovine serum (FBS)	ATCC	30-2020	
500 ml filter flask	Fisher	09-740-28C	
Neurobasal A medium	Invitrogen	12349015	
B27 supplement	Invitrogen	0080085SA	
Glutamax supplement	Invitrogen	35050061	
Zeocin	Fisher	NC9002627	
Blasticidin	Fisher	NC9016621	
BoNT/A holotoxin	Metabiologics	500 µg/ ml stock	
BoNT Resuspension Buffer	Metabiologics	NA	

P1000 sterile barrier tips	VWR	89092-094	
P200 sterile barrier tips	VWR	89092-108	
P20 sterile barrier tips	VWR	89092-106	
P10 sterile barrier tips	VWR	89092-104	
Multichannel sterile barrier tips	Gilson	DF300ST	
Sterile reagent bottles	Fisher	2019-0125	
Sterile 15 ml conical tube	ISC BioExpress	C-3394-2	
Sterile 50 ml conical tubes	VWR	89039-656	
Sterile reagent basins	ISC BioExpress	B-0812-2	
1.5 ml Eppendorf tubes	Fisher	05-402	

6. EQUIPMENT:

Equipment	Manufacturer	Model	BioSentinel Asset #
P1000 Pipetman	Gilson	P1000	multiple
P200 Pipetman	Gilson	P200	multiple
P20 Pipetman	Gilson	P20	multiple
P10 Pipetman	Gilson	P10	multiple
Pipet Aid	Drummond	C300	
Multichannel Pipetman	Gilson	C300 (F31014)	EQ1030q
Biological Safety Cabinet (BSC)	Labconco	Purifier Class II BSC	EQ 1017
Biological Safety Cabinet (BSC)	Labconco	Purifier BSC	EQ 1038
CO_2 Incubator	Thermo Scientific	Forma Series II	EQ 1016
CO_2 Incubator	Thermo Scientific	Forma Series II	EQ 1050
CO_2 Incubator	Thermo Scientific	Forma Series II	EQ 1039
Varioskan plate reader	Thermo	Varioskan Flash 3001	EQ1014
BioTek microplate washer	BioTek	ELx405	EQ1043

7. PROCEDURE:

7.1 Preparing cells prior to beginning experiment.

Note: Cells may be prepared according to other methods (e.g. ganglioside and/or RA treatment) than described in 7.1. Refer to specific SOP for cell preparation.

1. Passage cells as specified in SOP GL1033.
2. Count cells as specified in SOP GL1032.
3. Make MEM + 10% FBS cell growth medium.
    a. Pipet 50 ml ATCC fetal bovine serum into 500 ml MEM medium.
    b. Filter sterilize.
4. Add selection drugs to growth medium if required.
    a. Selection drugs are required for engineered cell lines, but not for original Neuro2A cell line.
    b. Zeocin and Blasticidin are required for BoCell A engineered cell lines.
    c. Add selection drugs to medium fresh each day. Discard remaining at the end of the day.

d. Use sterile barrier tips with all pipetmen.

e. Determine volume of medium required to plate cells.

f. Add appropriate volume 100 mg/ml stock Zeocin solution to a final working concentration of 600 ug/ml in MEM 10% FBS.

g. Add appropriate volume 10 mg/ ml stock Blasticidin solution to a final working concentration of 6 ug/ml in MEM 10% FBS.

5. Seed cells for one 96 well plate.

a. Make enough cell + medium mixture to fill 70 wells.

b. Seed 15,000 cells/ well in a volume of 100 ul/ well.

c. 70 wells x 15,000 cells per well= 1,050,000 cells required to seed one 96 well plate in a total volume of 7 mls MEM 10% FBS + selection drugs.

d. Mix cells and medium by pipetting up and down 10 times in a sterile 15 ml conical tube.

e. Refer to 96 well template at the end of this SOP. Seed only wells B3-G11. Do not seed cells into columns 1, 2, or 12. Do not seed cells into rows A or H.

f. Pipet cells + medium into a sterile reagent basin.

g. Set multichannel pipetman to speed 1, auto setting, 100 ul volume.

h. Seed 100 ul cells into each well.

i. Allow cells to settle for 20 minutes in plates while incubating in the biological safety cabinet at room temperature. Look at cells under the light microscope before 20 minute incubation. Cells should be uniformly distributed throughout well. If they are not, gently tip plate back and forth to redistribute cells.

j. Incubate plates in CO_2 incubator at 37 degrees, 5% CO_2 for 24 hours.

7.2 Incubation of cells with BoNT.

6. Make 100 mls Neurobasal A medium.

a. Thaw B27 and Glutamax supplements at 37 degrees until nearly thawed, then move to room temperature to complete thawing process.

b. Into a sterile 150 ml reagent bottle, pipet 97 mls of Neurobasal A medium, 2 mls B27 supplement, and 1 ml Glutamax supplement.

c. Mix well by pipetting up and down with a 25 ml pipet 3 times.

7. Determine how much Neurobasal A medium you are going to need for your particular experiment and add selection drugs. Make only what you need for the day, and discard remaining selection medium at the end of the day.

a. Pipet 25 mls above Neurobasal A medium into a sterile 50 ml conical tube.

b. Add 150 ul of 100 mg/ml Zeocin stock to a final concentration of 600 ug/ml.

c. Add 15 ul of 10 mg/ml Blasticidin stock to a final concentration of 6 ug/ml.

d. Mix well by pipetting up and down with a 25 ml pipet 3 times.

8. Using a multichannel pipetman, aim the pipet tips to the 6 o'clock edge of the wells. Remove 100 ul MEM 10% FBS medium from 3 rows of cells at a time. Being very careful not to allow cells to dry out, discard medium into a sterile reagent basin.

9. Using the multichannel pipetman, immediately pipet 100 ul/ well Neurobasal A medium with selection drugs made above.

10. Using a multichannel pipetman, remove 50 ul Neurobasal A medium with selection drugs from each well. You will end up adding 50 ul toxin mix to each well for a final total volume of 100 ul/ well.

11. Make 1:10, 1:100, and 1:1000 dilutions of BoNT/A 500 ug/ml stock into BoNT resuspension buffer. Prepare dilutions in sterile 1.5 ml Eppendorf tubes in the biological safety cabinet.

a. Make the 1:10 dilution first.
   i. Pipet 27 ul BoNT resuspension buffer into sterile 1.5 ml tube.
   ii. Add 3 ul BoNT/A toxin 500ug/ml.
   iii. Vortex briefly (1 sec) to mix.

b. Next make 1:100 dilution.
   i. Pipet 27 ul BoNT resuspension buffer into sterile 1.5 ml tube.
   ii. Add 3 ul 1:10 dilution of BoNT/A from above.
   iii. Vortex briefly (1 sec) to mix.

c. Finally make the 1:1000 dilution.
   i. Pipet 27 ul BoNT resuspension buffer into sterile 1.5 ml tube.
   ii. Add 3 ul 1:100 dilution of BoNT/A from above.
   iii. Vortex briefly (1 sec) to mix.

12. Make up toxin mixes at various concentrations listed in chart below. Make twice the volume of the 0nM concentration to be used in two separate columns- one column without cells as blanks, and one column with cells.

Toxin mixes for 1, 96 well plate (2x; add 50 ul per well for a final well volume of 100 ul)

BoNT/A Final Conc. (nM)	BoNT/A volume 500µg/ ml stock (3333 nM)	Metabiologics BoNT Resuspension buffer	Neurobasal A medium + selection drugs	Final volume
0	0 µl	16µl	734 µl	750 µl
0.01	2.25 µl of 1:1000 dil.	5.75 µl	367 µl	375 µl
0.03	6.75 µl of 1:1000 dil.	1.25 µl	367 µl	375 µl
0.1	2.25 µl of 1:100 dil.	5.75 µl	367 µl	375 µl
0.3	6.75 µl of 1:100 dil.	1.25 µl	367 µl	375 µl
1	2.25 µl of 1:10 dil.	5.75 µl	367 µl	375 µl
3	6.75 µl of 1:10 dil.	1.25 µl	367 µl	375 µl
10	2.25 µl	5.75 µl	367 µl	375 µl
30	6.75 µl	1.25 µl	367 µl	375 µl

Toxin mixes for 2, 96 well plates (2x; add 50 ul per well for a final well volume of 100 ul)

BoNT/A Final Conc. (nM)	BoNT/A volume 500µg/ ml stock	Metabiologics BoNT Resuspension buffer	Neurobasal A medium + selection drugs	Final volume
0	0 µl	30 µl	1470 µl	1500 µl
0.01	4.5 µl of 1:1000 dil.	10.5 µl	735 µl	750 µl
0.03	13.5 µl of 1:1000 dil.	1.5 µl	735 µl	750 µl
0.1	4.5 µl of 1:100 dil.	10.5 µl	735 µl	750 µl
0.3	13.5 µl of 1:100 dil.	1.5 µl	735 µl	750 µl
1	4.5 µl of 1:10 dil.	10.5 µl	735 µl	750 µl
3	13.5 µl of 1:10 dil.	1.5 µl	735 µl	750 µl
10	4.5 µl	10.5 µl	735 µl	750 µl
30	13.5 µl	1.5 µl	735 µl	750 µl

13. Using a multichannel pipetman, add 50 ul of toxin mixture to wells already containing 50 ul/ well Neurobasal A medium + selection drugs, for a total volume of 100 ul/ well.
14. Using a multichannel pipetman, add 100 ul/ well Neurobasal A medium + selection drugs to outer wells in rows A1-12, H1-12, columns 1, and 12. This prevents evaporation of medium from interior assay wells.
15. Incubate cells with BoNT/A for specified period of time at 37 degrees, 5% CO_2. Typical BoNT/A incubation time is 72 hours, but may be 48 or 24 hours.

7.3 Reading plate with Varioskan Flash microplate reader.

16. Following BoNT incubation, read plate on Varioskan Flash microplate reader EQ1014. 300 ms top read at the following wavelengths:
Excitation: 434nm, Emission: 470nm;
Excitation: 500nm, Emission: 527nm;
User Defined Equation: [value (Fluorometric 1 500/527)] / [value (Fluorometric1 434/470)]
17. PBS wash plate with BioTek microplate washer EQ1043 using protocol #15 (Cell WCT Nunc96 protocol) for Greiner 96 well plates containing cells.
18. Reread plate in Varioskan plate reader following PBS wash.
19. Print raw data for notebook.

7.4 Data analysis

20. Export data into Excel file.
21. Enter data into Prism file and graph the following.
    a. YFP Emission: RFU vs. BoNT/A concentration
    b. CFP Emission: RFU vs. BoNT/A concentration
    c. Ratio YFP Emission/ CFP Emission: Ratio (527/470) vs. BoNT/A concentration
22. Subtract background.

a. In Excel, average background values from blank wells containing no cells (column 2 as shown in sample plate diagram below).
b. Subtract appropriate background from each data set by using “analyze” and then “transform” options in Prism.
c. Enter transformed data back into Excel, and create a background subtracted ratio YFP-background/ CFP- background.

23. EC50 determination.
    a. Plot the background subtracted ratio YFP-background/ CFP- background against the negative log of the BoNT/A concentration.
    b. Remove the 0nM concentration and leave that value blank, however do not remove the data associated with that 0nM concentration.
    c. To determine EC50 values, select “analyze”, “nonlinear regression (curve fit)”, then “Sigmoidal dose-response (variable slope)”. Under “results” this will generate a table containing EC50 values.
24. Limit of Detection (LOD) determination.
    a. In Excel, average the values at each toxin concentration
    b. Determine the standard deviation (SD) of the 0nM concentration (control)
    c. Multiply the SD x3
    d. Calculate: control-3xSD to obtain the LOD

[BoNT/A]: blank 0nM 0nM .01 nM .03 nM .1 nM .3 nM 1 nM 3 nM 10 nM 30 nM

	1	2	3	4	5	6	7	8	9	10	11	12
A	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.
B	Med.	0nM	cells	cells	cells	cells	cells	cells	cells	cells	cells	Med.
C	Med.	0nM	cells	cells	cells	cells	cells	cells	cells	cells	cells	Med.
D	Med.	0nM	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Med.
E	Med.	0nM	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Med.
F	Med.	0nM	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Med.
G	Med.	0nM	cells	cells	cells	cells	cells	cells	cells	cells	cells	Med.
H	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.	Med.

Attachment 8.2: BoCell™ A Cell-Based Assay data

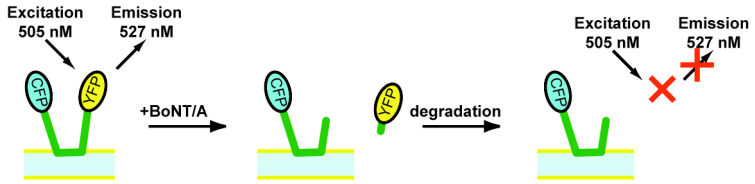


Figure 1. BioSentinel's BoCell™ A cell-based assay reporter. CFP and YFP are connected by SNAP-25 (green). SNAP-25 palmitoylation localizes the reporter to the plasma membrane. The YFP moiety is directly excited leading to fluorescence emission in the absence of BoNT/A. Cleavage of the reporter by BoNT/A releases a C-terminal reporter fragment containing the YFP moiety into the cytosol. The fragment is rapidly degraded and, thus, YFP emission is lost.

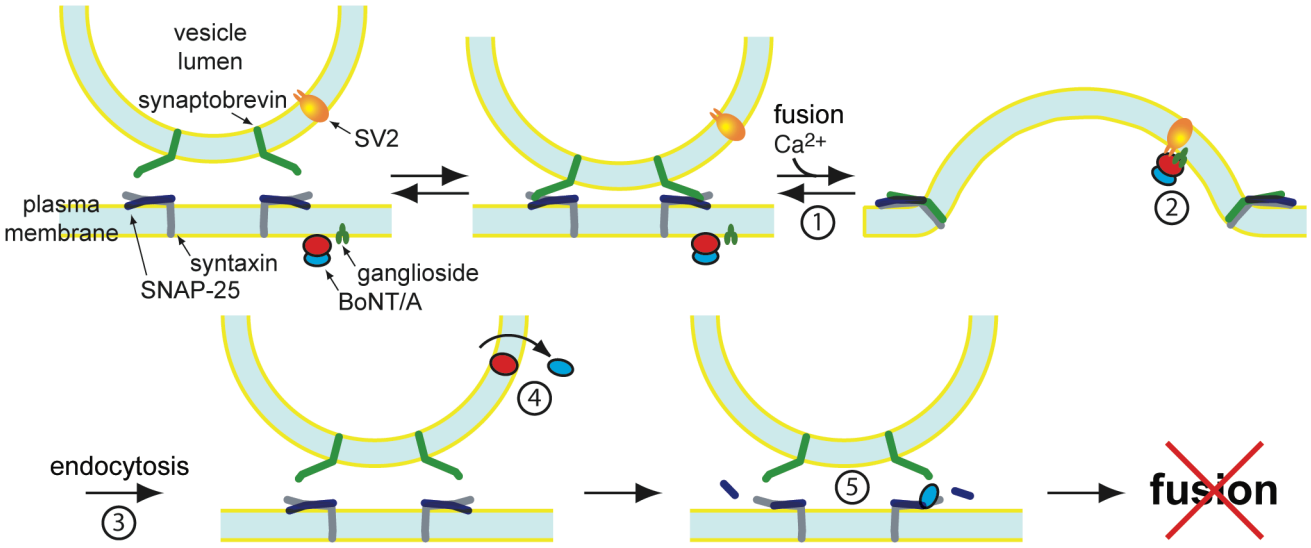


Figure 2. Mechanism of BoNT/A action. 1) Ca^{2+}-dependent membrane fusion is catalyzed by the SNARE proteins synaptobrevin, SNAP-25, and syntaxin; and the Ca^{2+}-sensor synaptotagmin (not shown). **2**) BoNT/A binds to the cell surface via interactions with gangliosides and a luminal loop domain of SV2, which is exposed at the cell surface after the vesicle fuses with the plasma membrane. **3**) BoNT/A is endocytosed along with the vesicle. **4**) Upon re-acidification of the vesicle lumen, the heavy chain (red) facilitates light chain translocation (cyan) into the cytosol. **5**) The light chain refolds and cleaves its target, SNAP-25 (or BioSentinel's BoCell™ reporter). Membrane fusion is disrupted, thus inhibiting the release of the vesicle's contents (i.e., neurotransmitter).

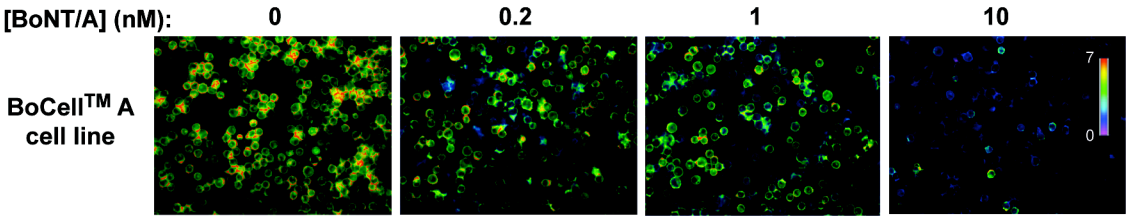


Figure 3. Fluorescence response of the BoCell™ A Cell-based assay. BoNT/A responses of the BoCell™ engineered cells. Cell were grown in 96-well plated, treated with the indicated BoNT/A concentration for 72 h, and imaged using fluorescence microscopy. Pseudo-colored images indicate the distribution the YFP/CFP fluorescence ratio within the cells.

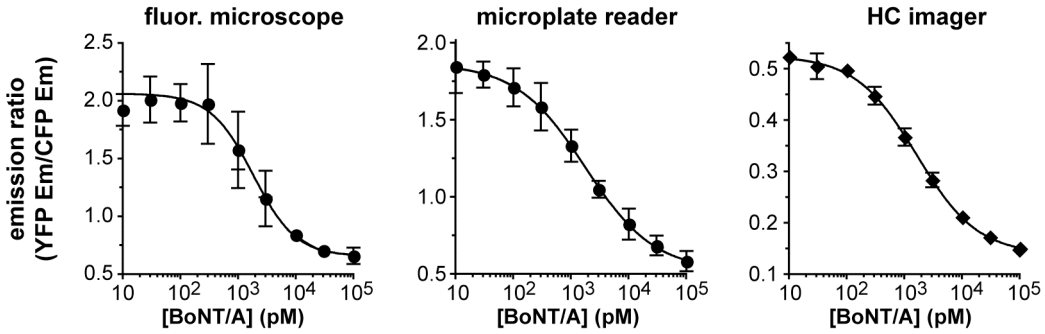
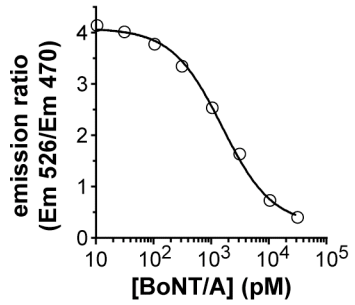


Figure 4. Performance of the BoCell™ Cell-based Assay using three different instruments to collect fluorescence emissions. Cells were plated and treated with BoNT/A according to the BoCell™ assay protocol (Attachment 8.1). YFP and CFP emissions were collected by microscopy (Nikon TE2000-U), fluorescence microplate reader (Thermo Varioskan), or bioimager (Molecular Devices ImageExpress). Emission rations were plotted as a function of BoNT/A concentration.

A



LOD = 39.5 pM (219 total mLD50 units)
LOQ = 133 pM (740 total mLD50 units)

B

[BoNT/A] (pM)	average emission ratio	SD	% CV	Z'-value
0	4.15	0.05	1.3	-
10	4.15	0.12	2.8	-232
30	4.03	0.09	2.2	-2.6
100	3.79	0.06	1.6	0.04
300	3.36	0.05	1.4	0.61
1000	2.55	0.06	2.4	0.78
3000	1.65	0.05	3.0	0.88
10000	0.74	0.08	10.4	0.88
30000	0.41	0.06	13.7	0.91

Figure 5. Performance of the BoCell™ A Cell-based Assay. The BoCell™ cells were titrated with BoNT/A using 12 replicates per BoNT/A concentration. (**A**) BoNT/A dose response of the BoCell™ A Cell-based Assay. Assay was completed as described in the attached protocol (Attachment 8.1) (**B**) Statistical performance of the BoCell™ A assay. Coefficients of variation (CV) were calculated by dividing the standard deviation by the average for the emission ratio values at a particular BoNT/A concentration. Z-factor values were calculated using the equation $Z' = 1 - [3x (\sigma_p + \sigma_n)] / |\mu_p - \mu_n|$, where σ is the SD, μ is the mean, p is the positive control (signal in the presence of the indicated BoNT/A concentration), and n is the negative control (signal in the absence of BoNT/A).

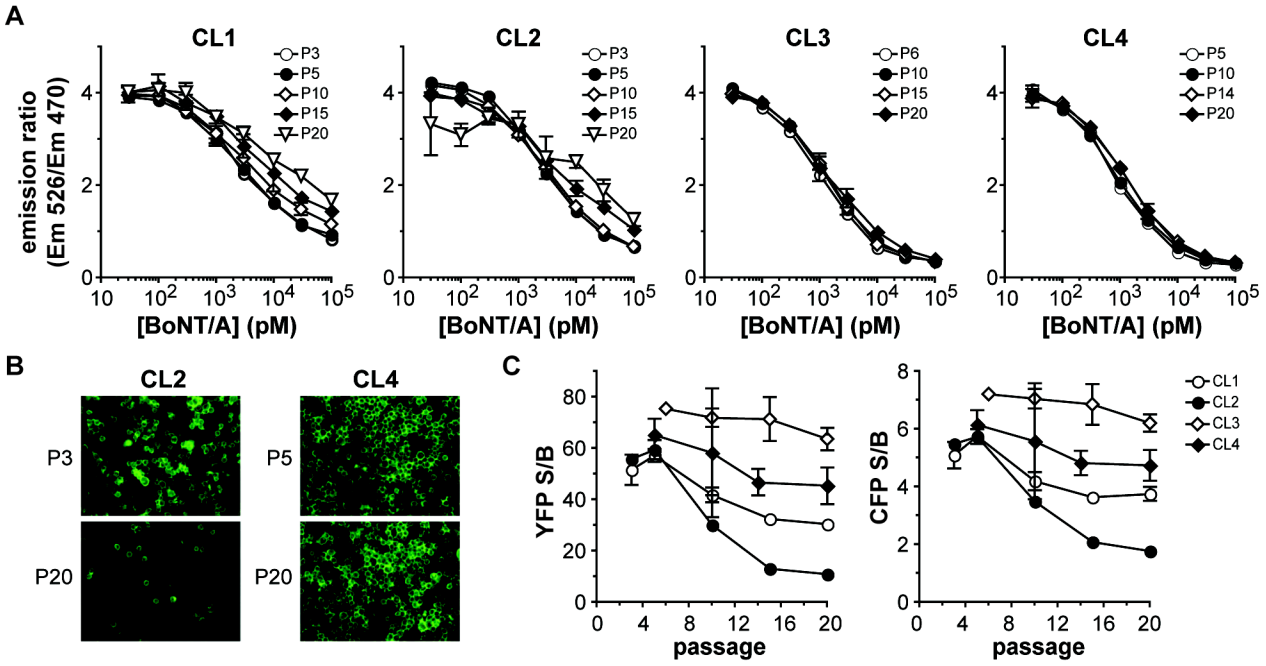


Figure 6. Passage stability of the candidate BoCell™ A cell-based assay cell lines. The indicated clones (CL1, CL2, CL3, and CL4) were passaged 20 times and cells were frozen at each passage. Cells from the indicated passages were then thawed and grown for an additional passage before being subjected to our BoCell™ A assay protocol (Attachment 8.1). (**A**) BoNT/A dose response of candidate cell lines. Each passage was subjected to varying concentrations of BoNT/A for 72 h before collecting YFP and CFP emissions with a microplate reader. Emission ratios were calculated by dividing the emission at 526 nm (YFP) by the emission at 470 nm (CFP). (**B**) YFP emissions of the CL2 and CL4 lines at varying passages. YFP emissions were collected by fluorescence microscopy. (**C**) YFP (left) and CFP (right) emission stability over passages. Emissions were collected with a microplate reader. Signal-to-background (S/B) is equal to the YFP or CFP emission of a control well containing cells but no BoNT/A divided by the YFP or CFP emission of a well containing no cells. Data in panels B and C were collected in the absence of BoNT.

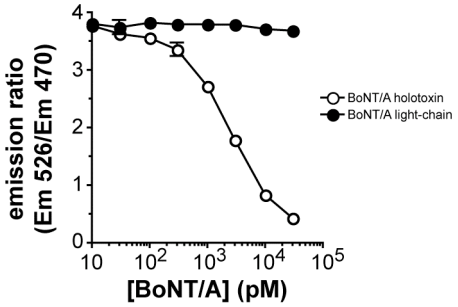


Figure 7. Only holotoxin is active in the BoCell™ A Cell-Based Assay. BoCell™ cells were subject to the BoCell™ A protocol (Attachment 8.1) in the presence of the indicated concentration of BoNT/A holotoxin or BoNT/A light chain. Emission ratios were plotted as a function of BoNT/A and fitted with equation Y=Bottom + (Top-Bottom)/(1+10$^{((LogEC_{50}-x)*Hill\ Slope)}$) to obtain EC_{50} values.

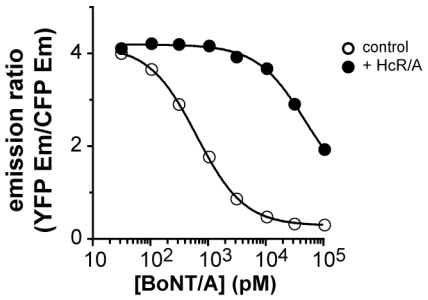


Figure 8. Blockade of BoNT/A activity in the BoCell™ A CBA using HcR/A. BoCell™ cells were subject to the BoCell™ A protocol (Attachment 8.1) in the presence and absence of 6 μM HcR/A. Emission ratios were plotted as a function of BoNT/A and fitted with equation Y=Bottom + (Top-Bottom)/(1+10$^{((LogEC_{50}-x)*Hill\ Slope)}$) to obtain EC_{50} values.